EFFECT OF VARIOUS LIPIDS ON CAROTENE STABILITY OF DEHYDRATED ALFALFA MEAL

by

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INTRODUCTION

The problem of carotene loss in dehydrated alfalfa during storage has been investigated intensively for many years (4). The need for an effective method of improving carotene stability is obvious in view of the nutritional importance of carotene as a vitamin A source for animals.

Enzymic activity is responsible for much of the carotene loss which occurs during drying of fresh alfalfa (8, 9, 15). It has been demonstrated, however, that in dehydrated alfalfa, enzymic destruction is not a factor (4, 16), and the loss appears to be due to autoxidation (3). The mechanism by which autoxidation takes place, and the oxidation products which form, have not been elucidated (4).

Several methods have been proposed for reducing the loss during storage. These include high moisture packing, use of antioxidants, and storage at low temperatures or under an inert atmosphere (4). The latter method is the most effective.

From earlier stabilization studies, it was established that the addition of small amounts of fats or oils to alfalfa meal resulted in improved carotene stability. The mechanism by which this occurs has not been elucidated. In the research reported below, the stabilizing ability of various lipids was studied in search of an explanation of this phenomenon.

REVIEW OF LITERATURE

While studying the effects of antioxidants on carotene stability, Kephart (11) suggested the use of oils as carriers for the application of antioxidants. He found that the oils made application easier and resulted in more uniform distribution of the antioxidant on the meal.

Thompson (24), in his studies with antioxidants, reported that some of the vegetable oils used as carriers had a small stabilizing effect when used alone, and that they reduced dustiness and enhanced the green color of the meal. Vitamin A and carotene stability in mixed feeds was found by Siedler and his co-workers (23) to be greater when stabilized animal fats were added than when no fat was added.

Mitchell, Beauchene, and Silker (14) found that carotene retention was influenced by the amount of oil used as a carrier. Similar results were obtained by Bickoff and his co-workers (2). Wesson Oil applied at a rate of 80 pounds per ton was more effective than at 16 or 32 pounds per ton (14). Heating the meal at 100° C. after application of the oil gave further stabilization, but no such response was obtained when the oil alone was heated before applying it to the meal (14).

Mitchell and Silker (17), and Ogden (18) studied the effect of various oils and fats. They found that animal fats gave greater stabilization, but were more difficult to apply.

Bickoff and co-workers (2), Ogden (18), and Denton and Bielanski (6) reported that carotene retention was adversely

affected by the addition of rancid fat, but when fresh fat was used, the addition of fat stabilizers gave no further improvement.

Ogden (18) found no relation between carotene retention and the degree of unsaturation or the molecular weight of added fats and oils. The presence of free fatty acids in vegetable oils did not affect their stabilizing ability, but when present in animal fats, a detrimental effect was observed.

Bickoff and his co-workers (2) found that fats and oils containing from 5 to 45 percent free fatty acids had a similar effect on carotene stability and color retention as those having low free fatty acid contents. Ogden (18) found no correlation between stabilizing ability and melting points of the fats. He also found that addition of more than 5 percent of an oil gave no further improvement.

It has been suggested that the increased carotene retention obtained when oils or fats are added, and the enhancement obtained by heating, are due to better penetration of the antioxidant through the protein portion of the meal (14). The stabilizing effect of the oil alone could be due to mutual solution of the carotene and the natural antioxidants present in the meal (2, 18). Native antioxidants could also be present in the oils, which might account for their stabilizing effect (18). If solubility factors are involved, variations in the polarity of the oiling agents should influence carotene retention.

Investigations also have been made using purified carotene in solution. They indicate that carotene is more stable in oil

solutions (22), and that an increase in the peroxide number of the oil reduces its potency as a stabilizer (20, 21).

EXPERIMENTAL AND RESULTS

The fatty acids used in these experiments were purchased from various commercial sources, except ricinoleic acid, which was prepared by saponification of commercial methyl ricinoleate by the Hilditch method (12).

Methyl and ethyl esters of the fatty acids were synthesized by direct esterification of the alcohols with the acids as indicated by Gunstone (7). The extent of esterification was determined chromatographically, using a tricalcium phosphate-Supercel adsorbent mixture as described by Dalton and Mitchell (5). Half gram samples of the esters were dissolved in Skellysolve B, and adsorbed on 10-inch columns of the adsorbent. On washing the column with Skellysolve B, the ester was eluted while the free acid and alcohol remained on the column. The octanoate and laurate esters did not elute sharply from the column, and it was necessary to estimate the free acid content of these preparations by titration with a standard base solution. All the ester preparations, as finally used, contained only traces of free acid.

The method of Wheeler, Riemenschneider, and Sando (26) was used for the synthesis of tristearin, except that reaction temperatures were 140-150° C. as suggested by Holman, Malkin, and Lundberg (10). The method consisted of heating a mixture of glycerol and stearic acid in the presence of 2 percent

p-toluenesulfonic acid. A 6 percent excess of the stearic acid was used to insure complete esterification. The catalyst was prepared as indicated by Vogel (25).

Mixed monoglycerides were prepared from Wesson Oil by the method of Mattil and Sims (13). One part of Wesson Oil, four parts of glycerol, and eight parts of pyridine were heated at 100° C., in the presence of sodium methylate, until a homogeneous solution was obtained. The reaction mixture was then transferred to a separatory funnel, and dilute hydrochloric acid was added to quench the catalyst. Pyridine hydrochloride was removed by washing with a saturated salt solution. The monoglyceride content of the preparation was determined by the method of Quinlin and Weiser (19), which consists of dissolving one gram of the sample in chloroform, adsorbing the lipid on a column of silicic acid, and eluting first with benzene to remove the triglycerides, then with 20 percent ether in benzene to elute diglycerides, and finally with ether to elute monoglycerides. The monoglyceride content was found to be about 75 percent by this method.

The various lipids were applied to alfalfa meal at rates of 1 and 4 percent (based on the weight of alfalfa used) as follows: 200 grams of commercially dehydrated alfalfa meal were placed in a rotary mixer. The lipids were dissolved in about 25 ml of a suitable volatile solvent and the solutions were delivered to the meal from a pipet while the mixing chamber was rotated at 12 r.p.m. The oil meal was thoroughly mixed for 5-10 minutes and then was spread on a paper, where it was allowed to dry for at

least two hours at room temperature. The samples were mixed and placed in 4-oz. bottles, and the carotene content of each was determined by the A.O.A.C. method (1). The bottles then were placed in an incubator operating at 36° C., and carotene determinations were made again after four weeks of storage.

Carotene Stability of Alfalfa Meals

The effect of chain length of the hydrocarbon portion of a lipid was studied with certain fatty acids and their methyl esters. The results are presented in Tables 1 and 2. Carotene stability of untreated meal and of meal treated with Wesson Oil is included for comparison. A portion of each sample was heated for an hour at 100° C. prior to storage, because earlier work had shown that this treatment enhanced the effectiveness of some lipids.

Table 1. Carotene stability in alfalfa meal treated with methyl esters of fatty acids of varying chain length.

Material added	Amount %	Percent carotene loss in four weeks	
		Unheated	Heated
None	--	44	40
Wesson Oil	1	44	38
	4	30	24
Methyl octanoate	1	44	38
	4	38	33
Methyl laurate	1	45	34
	4	24	26
Methyl stearate	1	44	33
	4	26	20

Table 2. Effect of free fatty acids on carotene stability in alfalfa meal.

Material added	Amount %	Percent carotene loss in four weeks	
		Unheated	Heated
None	--	51	51
Wesson Oil	1	52	46
	4	39	36
Octanoic acid	1	50	50
	4	48	36
Lauric acid	1	52	48
	4	42	32
Stearic acid	1	45	47
	4	--	--

In general, application of 1 percent of the lipid did not appreciably affect carotene retention. Addition of 4 percent of the esters resulted in an appreciable increase in stability, and there was a further increase when the latter samples were heated. The stabilizing effect was greater with increasing fatty acid chain length. A combination of 4 percent methyl stearate and heat treatment produced the greatest carotene stability. With the free fatty acids, the additional benefit derived from the higher rate of application was less than was obtained with the methyl esters.

These data show that, although increases in carotene stability can be achieved by treating meal with free fatty acids and subsequently subjecting it to heat, the benefit obtained is less than can be obtained by using fatty acid esters. The higher rate

of application of stearic acid is not easily achieved because it is not sufficiently soluble in the solvents used for applying it to the meal. This accounts for the lack of data for stearic acid in Table 2.

In addition to their lesser ability to stabilize carotene, free fatty acids differed from their methyl esters in their effect on the color of the meal. The methyl esters had little effect on color, while the fatty acids caused development of a brown color due to the conversion of chlorophyll to pheophytin.

The influence of a change in the alcohol portion of the esters of a given fatty acid is shown in Table 3. The methyl and ethyl esters of stearic acid greatly improved carotene stability at the higher rate of treatment, and were essentially equal in their effect. Tristearin was essentially ineffective. Methyl stearate and ethyl stearate melt at a temperature slightly above room temperature, while tristearin remains hard. It seemed probable that tristearin failed to penetrate into the alfalfa particles and therefore was unable to exert an influence.

If penetration of a lipid is a factor in its ability to improve carotene stability, the presence of free alcohol groups in the lipid might facilitate its penetration through the proteins and polysaccharides of the meal. To test this postulate, experiments were performed with ricinoleic acid (12-hydroxyoleic acid) and its esters, and with glycerol and monoglyceride. The data are presented in Tables 4 and 5.

Table 3. Variation in carotene stability of alfalfa samples with changes in the alcohol portion of an ester.

Material added	Amount %	Percent carotene loss in four weeks: Unheated	Heated
None	--	48	42
Wesson Oil	1	46	38
	4	34	27
Methyl stearate	1	44	33
	4	26	20
Ethyl stearate	1	44	32
	4	29	22
Tristearin	1	49	44
	4	40	41

Table 4. Influence of the presence of free polar groups on the stabilizing power of the oiling agents used on alfalfa.

Material added	Amount %	Percent carotene loss in four weeks: Unheated	Heated
None	--	48	46
Wesson Oil	1	48	42
	4	34	30
Methyl oleate	1	46	34
	4	24	24
Methyl ricinoleate	1	41	32
	4	36	22
Oleic acid	1	50	46
	4	41	40
Ricinoleic acid	1	50	46
	4	47	38

Table 5. Relative carotene stabilizing effect of glycerol and glycerides on dehydrated alfalfa.

Material added	Amount %	Percent carotene loss in four weeks	
		Unheated	Heated
None	--	52	51
Wesson Oil	1	48	46
	4	36	32
Monoglyceride	1	45	42
	4	46	38
Glycerol	1	56	53
	4	50	48

Methyl oleate and methyl ricinoleate were quite similar in their stabilizing abilities, and so were oleic and ricinoleic acids. Again the free acids were essentially ineffective, even at the higher rate of application and after heating. The presence of free alcohol groups on the glycerol portion of the added substances did not further improve stabilizing ability. The activity of the monoglyceride prepared from Wesson Oil was not appreciably different from that of Wesson Oil itself, and actually showed less improvement on heating than Wesson Oil. Glycerol was devoid of stabilizing activity. These data indicate that the free alcohol groups do not improve the activity of the lipids.

Since it is known that highly unsaturated lipids autoxidize more rapidly than less unsaturated lipids, and since the products of autoxidation catalyze the oxidation of other compounds, it was thought that the degree of unsaturation of an oiling agent might influence its stabilizing ability. This was studied with stearic,

oleic, and linoleic acids and their esters, with the results shown in Tables 6 and 7.

Table 6. Effect of unsaturation on the carotene stabilizing ability of methyl esters.

Material added	Amount %	Percent carotene loss in four weeks: Unheated	Heated
None	--	44	40
Wesson Oil	1	44	38
	4	30	24
Methyl stearate	1	44	33
	4	26	20
Methyl oleate	1	46	34
	4	24	24
Methyl linoleate	1	48	36
	4	46	25

Table 7. Carotene stability of alfalfa meals treated with unsaturated fatty acids.

Material added	Amount %	Percent carotene loss in four weeks: Unheated	Heated
None	--	51	51
Wesson Oil	1	52	46
	4	39	36
Stearic acid	1	45	47
	4	--	--
Oleic acid	1	50	46
	4	41	50
Linoleic acid	1	50	46
	4	44	37

Although the value for the unheated sample containing 4 percent methyl linoleate appears to be erroneous, the activities of the three esters and Wesson Oil were quite similar. However, a marked decrease in the initial carotene content of the methyl linoleate-treated meal was always noted when compared to the initial values for untreated meal (Table 8). No other oiling agent studied had such an effect. When "percent loss in four weeks" was calculated, using the initial value of the untreated meal, it was clear that meal treated with methyl linoleate lost much more carotene than meals treated with the other oiling agents. The reason for this behavior is obscure.

Table 8. Effect of linoleic acid and methyl linoleate on the initial carotene content of alfalfa meal.

Material added	Amount %	Carotene content, mg. per 100 g. Unheated	Heated
Untreated	-	24.4	24.4
Wesson Oil	1	24.2	24.0
	4	24.0	23.2
Methyl linoleate	1	21.2	20.8
	4	18.5	16.6
Linoleic acid	1	23.0	22.7
	4	20.0	19.5

When the free acids were used, no distinct difference between them was noted. Again, an initial carotene loss was observed with linoleic acid, similar to that associated with the use of methyl linoleate. The data indicate that mono-unsaturation is not detrimental to the stabilizing action of the lipid,

but that the presence of di-unsaturation is undesirable.

Stability of Carotene Concentrates

Some of the data discussed earlier indicated that penetration of the lipids into the alfalfa particles might be a factor influencing their stabilizing activity. This factor was eliminated by extracting carotene and associated lipids from the meal and observing the effectiveness of the oiling agents when added to concentrates prepared from the alfalfa extracts.

Carotene was extracted by soaking four to five pounds of dehydrated alfalfa in successive portions of Skellysolve B for two to three hours. Powdered tricalcium phosphate was added to the extract until all xanthophylls and chlorophyll were adsorbed and a bright yellow solution remained. A carotene determination was made at this stage to check for complete removal of other pigments and to determine how much the solution needed to be concentrated. Excess Skellysolve B was removed by evaporating on a steam bath until the desired volume was attained. The final concentrate had a carotene content such that when 10 ml. of it were added to 50 grams of cerelose (to be used as carrier), the carotene concentration of the solid mixture was 25-30 mg./100 g., which is the usual concentration in most dehydrated meals.

Ten ml. of the concentrate, 50 g. of cerelose, and the proper amount of a solution of the lipid to be tested were thoroughly mixed in a 600 ml. beaker until the yellow color was evenly distributed. The mixture was transferred to a paper and mixed

further to assure uniformity, and then was allowed to dry at room temperature for about two hours. After the initial carotene determination was made, the concentrates were incubated at 36° C. and were analyzed for carotene after 3, 7, 10, and 14 days. At 14 days, the untreated concentrates had lost about the same percent of the carotene as the untreated alfalfa samples lost in four weeks.

The results obtained with the concentrates are shown in Table 9.

In general, the results were similar to those obtained with alfalfa meal. Methyl laurate, methyl stearate, and methyl oleate were better stabilizers than methyl octanoate, but the esters were, in general, less effective in the concentrates than Wesson Oil. Here again, the free fatty acids made carotene less stable than did their methyl esters. While methyl oleate improved stability, methyl ricinoleate was ineffective. The higher rate of application of most of the esters tended to increase stability. The detrimental effect of methyl linoleate and linoleic acid was much more apparent with the concentrates than with the meals.

DISCUSSION

The data obtained with the concentrates do not unequivocally support the belief that penetration is a factor influencing the ability of an oiling agent to improve carotene stability. Although the higher level of esters applied without heat to the meal were slightly better than Wesson Oil, corresponding treatment

Table 9. Stability of carotene concentrates treated with various fatty acids and their methyl esters.

Material added	Amount %	Percent carotene loss: Days of storage 3	7	10	14
None	--	23	34	46	64
Wesson Oil	1	10	14	22	30
	4	6.4	8.2	12	18
Methyl octanoate	1	28	38	48	59
	4	17	24	34	43
Methyl laurate	1	17	21	33	46
	4	7.0	7.7	13	16
Methyl stearate	1	15	24	32	42
	4	9.0	16	24	29
Methyl oleate	1	17	24	35	47
	4	7.0	9.6	12	18
Methyl linoleate	1	23	38	67	100
	4	27	70	100	--
Methyl ricinoleate	1	28	38	58	72
	4	17	38	44	60
Octanoic acid	1	16	24	33	44
	4	22	41	64	89
Lauric acid	1	28	34	43	52
	4	28	31	40	46
Stearic acid	1	22	39	48	58
	4	27	46	55	61
Oleic acid	1	14	22	30	38
	4	14	23	30	39
Linoleic acid	1	33	48	63	79
	4	60	86	100	--
Ricinoleic acid	1	30	40	49	58
	4	40	56	68	78

of the concentrates showed little difference between the esters (other than methyl octanoate) and Wesson Oil. This would indicate that penetration was a factor. However, the differences observed were not great enough to assure significance. Any such comparison is complicated by the fact that Wesson Oil contains natural antioxidants which might contribute to stability also. Increased stability following heating of the oiled meals still is the most impressive evidence that penetration is a factor.

The results of this study show definitely that free fatty acids are less desirable than Wesson Oil as oiling agents. This is especially apparent in the case of linoleic acid supplied to carotene concentrates. This might explain earlier results (17) that acidified cottonseed soapstock was ineffective as a carotene stabilizer for the alfalfa meal, since this material contains an appreciable amount of linoleic acid.

The importance of the quality of the oiling agents has decreased recently because of a technological development. Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), the approved antioxidant for addition to dehydrated alfalfa, now is available in a water emulsifiable form. The present practice is to apply this preparation to the meal, pass the meal through a pelleting machine, and store the resulting pellets. Dustiness thus is not a problem, and oil need not be added. However, not all of the dehydrated alfalfa produced in this country is converted to pellets. It therefore is still desirable to use the oil soluble form of the antioxidant to control dustiness and to

improve stability when dehydrated alfalfa is to be stored as meal. The producers of meal thus must still be concerned about the quality of the oiling agents employed.

SUMMARY

Several lipids were synthesized, and their effect on carotene stability in dehydrated alfalfa and in carotene concentrates was determined. The greatest stability was obtained with the lower alcohol esters (methyl and ethyl) of fatty acids having long hydrocarbon chains. The presence of free hydroxyl groups did not improve stabilizing effectiveness. Free fatty acids were not good stabilizers. Heating the oiled alfalfa samples resulted in enhanced effectiveness in most instances.

None of the lipids tested, either with the meal or with the concentrates, gave much better preservation than Wesson Oil, which was always used for comparison.

The results indicate that the chemical nature of a lipid influences its carotene stabilizing ability.

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The problem of carotene loss in dehydrated alfalfa during storage has been investigated intensively for many years. From these studies it was established that the addition of small amounts of fats or oils improved carotene stability. However, some oiling agents appeared to be more effective than others. The purpose of this research was to study the relation of chemical structures of lipids to their stabilizing ability.

Octanoic, lauric, stearic, linoleic, and oleic acids were purchased from commercial sources. The methyl, ethyl, and glycerol esters were synthesized by heating the fatty acids with the corresponding alcohol in the presence of a catalyst. Ricinoleic acid was obtained by saponification of methyl ricinoleate, which was obtained from a commercial source. A preparation having a high monoglyceride content was made by glycerolysis of Wesson Oil.

The lipids were applied to commercially dehydrated alfalfa meal at the rates of 1 and 4 percent, based on the weight of alfalfa used. Alfalfa samples were placed in a rotary mixer and the lipids, dissolved in suitable volatile solvents, were delivered from a pipet onto the rotating meal. After thorough mixing, and allowing the solvent to evaporate, the samples were stored in 4-oz. bottles. Untreated meal and samples treated with Wesson Oil also were studied for comparison. After determining the initial carotene content, the samples were incubated at 36° C., and were analyzed for carotene again after four weeks.

To study the influence of penetration of the lipids on carotene stabilizing ability, a portion of each oiled sample was heated at 100° C. for one hour and subjected to the stability test. Also, the effect of some of the lipids on carotene concentrates was determined. Solid concentrates were prepared by mixing the lipids and carotene (extracted from alfalfa) on 50 grams of cerelose. The concentrates contained about 25 mg. percent of carotene and added lipids at the rate of 1 and 4 percent, similar to the alfalfa samples. Carotene was determined at zero time, and after 3, 7, 10, and 14 days of incubation at 36° C.

The best stability was obtained with the methyl esters of fatty acids of long hydrocarbon chains. Free fatty acids were not effective as stabilizers. Mono-unsaturation in the oiling agent did not alter carotene stability, but poly-unsaturation appeared to be undesirable. Incorporation of free hydroxyl groups into the lipid molecule did not improve its carotene stabilizing potency. The stabilizing ability of the most effective methyl esters was similar to that of Wesson Oil. Heating the oiled samples resulted in additional increases in carotene stability. Studies with the carotene concentrates showed the same general trends as were obtained with the alfalfa meal. However, the degree of stabilization produced by the oiling agents was comparatively less with the concentrates.

The results of this work indicate that the chemical nature of a lipid used as an oiling agent has some influence on carotene stability. The increased stabilization obtained by heating the

oiled samples is still the strongest evidence favoring the idea that penetration of oiling agents into the meal particles, to provide a better contact between carotene and antioxidants, is responsible for the carotene stabilizing effect of certain lipids, but otherwise the data do not give a definite confirmation of this possibility.